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Manganese Chloride Enhances Murine Cell-Mediated Cytotoxicity Effects on Natural Killer Cells

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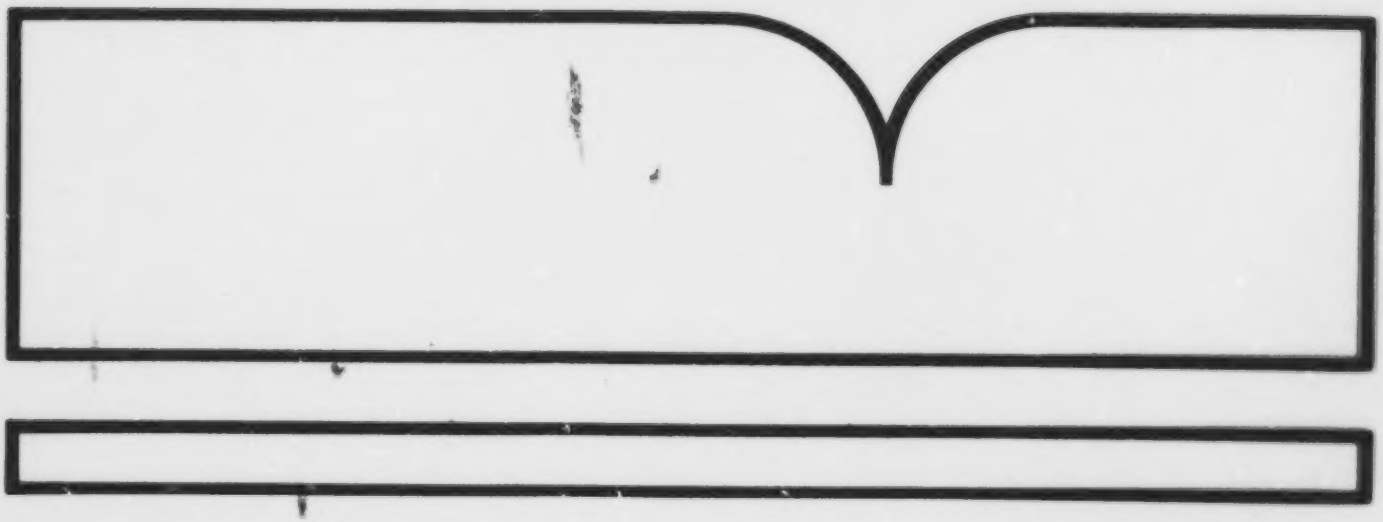

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16. ABSTRACT

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MANGANESE CHLORIDE ENHANCES MURINE CELL-MEDIATED CYTOTOXICITY: EFFECTS ON NATURAL KILLER CELLS

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ABSTRACT

Natural killer (NK) cell activity of mice given a single injection of manganese chloride ($MnCl_2$) was significantly enhanced as measured in a 4-hr *in vitro* ^{51}Cr release assay. Enhanced activity persisted for several days after injection. This cytotoxic activity was associated with nonadherent spleen cells and was completely eliminated by injecting $MnCl_2$-treated mice with anti-asialo GM_1 serum. Manganese-enhanced natural cytotoxicity was observed in several mouse strains with differing NK cell reactivity (CBA/J, C57BL/6, A/J, C3H/HeJ, and C57BL/6 beige mice) and with several tumor target cells with differing sensitivity to NK cytolysis (YAC-1, RBL-5, EL-4, and P815). The growth of B16-F10 melanoma lung tumors was inhibited in mice injected with $MnCl_2$ one day before tumor challenge. Manganese chloride enhancement of NK cell activity appeared to be mediated by interferon (IFN). Low levels of IFN were detected in the serum of mice as early as 4 hr after $MnCl_2$ injection. Rabbit anti-mouse IFN α,β but not anti-mouse IFN γ completely eliminated the $MnCl_2$-enhanced NK cell activity in the spleens of mice. The observed enhancement of NK cell activity by $MnCl_2$ is similar to that reported for more complex molecules that act by inducing IFN production.

INTRODUCTION

Natural killer (NK) cells display spontaneous *in vitro* and *in vivo* cytotoxic reactivity against syngeneic and allogeneic tumor

cells without immunologic priming and therefore have been suggested as one of the first lines of defense against newly arising tumors (1-5). Several studies suggest a possible role for NK cells as *in vivo* effectors against transplanted tumors (6-8).

Murine NK cell activity can be regulated by various bacterial, viral and synthetic agents. Several of these agents enhance the production of interferon (IFN), which in turn stimulates NK cell activity (9-11).

In recent work, we observed that injection of nickel chloride ($NiCl_2$) caused a suppression of murine splenic NK cell activity (12), whereas manganese chloride ($MnCl_2$) augmented NK activity after a single intramuscular injection (13). The present study further examines the effect of $MnCl_2$ on NK cell activity and the role that IFN plays in the enhancement of natural cell-mediated cytotoxicity (CMC). The results show 1) that $MnCl_2$-enhanced CMC is associated with a nonphagocytic, nylon wool nonadherent cell population that is inactivated by anti-asialo GM. serum, which indicates that NK cells are the effector cells and 2) that IFN α,β mediates $MnCl_2$ enhancement of NK cell activity.

MATERIALS AND METHODS

Mice: Eight-week-old female (C57BL/6J and C57BL/6J-Bg^{r}) and male (CBA/J, C3H/HeJ, and A/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in our animal facilities for at least 1 wk after arrival to allow recovery from shipping.

Manganese Chloride: A stock solution of $MnCl_2$ ($MnCl_2 \cdot 4H_2O$, Fisher Scientific Co., Fair Lawn, N.J.) was prepared in sterile, nonpyrogenic, normal (0.9%) saline. The manganese concentration was determined by neutron activation analysis (Dr. J.N. Weaver, North Carolina State University, Raleigh, NC). For injection, aliquots of the $MnCl_2$ stock solution were diluted to the desired concentration in sterile saline buffered with 25 mM 2-N-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES, Grand Island Biological Co., Grand Island, N.Y.). Unless stated otherwise, the mice were given a single intramuscular injection in the thigh on a body weight basis with injection volumes ranging from 0.10 to 0.15 ml. Control mice were injected with equivalent volumes of HEPES-saline.

IFN Inducers: Polyinosinic:polycytidylic acid (poly I·C, Grand Island Biological Co., Grand Island, NY) and tilorone hydrochloride (kindly provided by J.M. Cretsos, Merrel Dow Pharmaceuticals, Inc., Cincinnati, OH) were dissolved in saline. The mice were given 0.1 mg poly I·C intravenously 18 hr before testing or 2 mg tilorone by oral gavage 24 hr before testing.

IFN Assay: IFN (α,β,γ) levels in mouse serum were determined by inhibition of the cytopathic effect of Vesicular Stomatitis Virus on murine L929 cells by Biofluids, Inc., (Rockville, MD).

Antisera: Rabbit anti-mouse IFN α,β (lot #2/04/001) with 2.8×10^4 neutralizing units/ml and rabbit anti-mouse IFN γ (lot #2/09/001) with 5.0×10^4 neutralizing units/ml (NIH reference) were obtained from Enzo Biochem, Inc., (New York, NY).

Rabbit anti-asialo GM_1 (lot #PER-9413) with 10 mg protein/ml was obtained from Wako Chemicals USA, Inc., (Dallas, TX).

Preparation of Effector Cells: Spleen cell suspensions were prepared in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO). Erythrocytes were lysed by adding 0.83% NH_4Cl. Cell suspensions were washed and resuspended to 2 X 10^7 viable cells/ml. Viability was determined by trypan blue exclusion.

Tumor Cell Lines: Four in vitro murine tumor cell lines were used as targets in the cytotoxicity test: YAC-1 from a Moloney virus-induced lymphoma in A/Sn mice (obtained from Dr. A. Burger, Duke University, Durham, NC); P815 from a methylcholanthrene-induced mastocytoma cells in DBA/2 mice (obtained from Dr. G.Y. Gillespie, University of North Carolina, Chapel Hill, NC); EL-4 from a benzo[a]pyrene-induced lymphoma in C57BL/6 mice (obtained from Dr. D. Adams, Duke University); and RBL-5 from a Raucher virus-induced lymphoma in C57BL/6 mice (obtained from Dr. H.T. Holden, Frederick Cancer Research Center, Frederick, MD). All cell lines were grown as suspension cultures in RPMI/FBS (10%) supplemented with antibiotics (100 IU/ml of penicillin, 100 μg/ml of streptomycin). Logarithmically growing cells were used in cytotoxicity tests.

In Vitro Cytotoxicity Assay: Target cells were labeled with ^{51}Cr (50 μCi ^{51}Cr as sodium chromate/10^6 cells, specific activity 200 to 500 Ci/g, New England Nuclear, Boston, MA) for 1 hr at 37°C.

The cells were washed three times in RPMI/FBS and resuspended to 2×10^5 cells/ml. Labeled target cells (2×10^4 cells/100 µl) were cultured with graded numbers of splenic effector cells (100 µl) in round-bottom microtiter plates (Linbro, Flow Labs, Rockville, MD). Three replicates of each effector-target cell ratio (25:1, 50:1, and 100:1) were incubated for 4 hr at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After incubation, the plates were centrifuged for 5 min at 250 x g, and the supernatant was collected using the Titertek supernatant collection system (Flow Labs). Percent specific ^{51}Cr release was determined from the formula:

$$[(E-S)/(T-S)] \times 100$$

where E is the ^{51}Cr released from target cells in the presence of spleen cells, S is the spontaneous release of ^{51}Cr from target cells alone, and T is the maximum release of ^{51}Cr from target cells in the presence of 0.25% Triton X-100. Spontaneous release was typically less than 10% of the maximum releasable ^{51}Cr activity.

Removal of Phagocytic and Nylon Wool-Adherent Cells:

Phagocytic cells were removed from spleen cells suspensions pooled from 5 mice by adding 10 ml of 1×10^7 cells/ml to tubes containing 1 g of carbonyl iron (CI, GAF Corporation, New York, NY). Cells were incubated at 37°C, gently mixed every 5 to 10 min, and then the cells associated with CI were removed by multiple passage over a magnet (11). The cells remaining in suspension were then passed over nylon wool columns (NW) as described by Marosan et al. (14). Briefly, 0.6 g of nylon wool (Fenwal Laboratories, Deerfield, IL)

was placed into the barrel of a 10-ml syringe. The NW was washed with 20 ml of warm RPMI/FBS medium, and 1 x 10^6 CI-fractionated cells in 1 ml of RPMI/FBS medium were added drop-wise to the column. The columns were incubated for 1 hr at 37°C in 5% CO_2. After incubation, the cells that did not adhere to the nylon wool were collected and washed with 10 ml of warm medium and resuspended to 2 x 10^7 cells/ml.

B16-F10 Tumors: The development of B16-F10 lung tumors in mice following $MnCl_2$ treatment was determined as described by Hanna and Burton (7). B16-F10 melanoma tumor cells (obtained from Dr. I. Fidler, National Cancer Institute, Frederick, MD) were grown in monolayer cultures maintained in Eagle's minimum essential medium (MEM) supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution (CMEM, GIBCO). Exponentially growing cells were harvested from cultures by overlaying monolayers with a solution of 0.25% trypsin-0.02% EDTA for 1 min. The cells were washed in CMEM and resuspended in MEM to 5 x 10^5 cells/ml. Unanesthetized C57BL/6J mice were injected intravenously with 0.2 ml of B16-F10 cells. The mice were killed 2 to 3 wk later, and their lungs were removed and fixed in Bouin's solution. Pulmonary tumor colonies were counted with the aid of a magnifying glass.

Statistical Analysis: Data from the in vitro cell assay were analyzed using Student's t-test or Dunnett's t-test (2-tailed). The nonparametric Mann-Whitney U test (2-tailed) was used to analyze the B16-F10 lung tumor data.

RESULTS

Data in Table 1 demonstrate the enhancing effect of a single intramuscular $MnCl_2$ injection on CMC in several mouse strains. Significant enhancement of CMC against YAC-1 targets in high (CBA/J), intermediate (C57BL/6J and C3H/HeJ), and low (A/J and C57BL/6J-Bg^r) NK reactive mouse strains was observed 24 hr after a single 80 μg/g $MnCl_2$ injection. Natural cellular cytotoxicity was enhanced at $MnCl_2$ doses of 10 to 160 μg/g depending upon the strain used, with no difference in the number of nucleated cells per spleen between saline- and $MnCl_2$-treated mice (data not shown).

Table 2 shows that $MnCl_2$ significantly ($P < 0.01$) enhanced CMC in C57Bl/6J mice against a variety of target cells. This enhancement was observed at all effector:target cell ratios.

The effects of different $MnCl_2$ injection routes on splenic CMC were determined (Table 3). Although all parenteral routes were effective, the intravenous route was the most effective, with comparable enhancement at one-fourth the dose required by the intramuscular, intraperitoneal, and subcutaneous routes. The oral route, however, was not effective even at a dose four times that of the intramuscular route.

The cells responsible for $MnCl_2$-enhanced CMC were characterized by removing phagocytic and nylon wool-adherent cells or treating mice with rabbit anti-asialo GM_1. The cytotoxic activity of spleen cells from saline- and $MnCl_2$-injected C57BL/6J mice before and after CI or CI-NW was examined (Table 4). The results show that although CI and CI-NW treatment of spleen cells

TABLE 1

The Effect of $MnCl_2$ on NK Cell Activity of Different Mouse Strains

	$\bar{x}$ % ^{51}Cr Release ± SE[a]					
	Saline E:T Ratio			$MnCl_2$ (80 μg/g)[b] E:T Ratio		
Strain	25:1	50:1	100:1	25:1	50:1	100:1
CBA/J(H-2^k)	13.5±1.3	19.0±0.6	21.3±1.3	24.7±1.8	36.5±2.8	44.8±3.0
C3H/HeJ(H-2^k)	3.7±0.2	4.8±0.3	3.1±0.3	14.3±0.9	20.6±1.3	21.3±1.5
C57BL/6J(H-2^b)	6.5±0.4	9.6±0.5	13.2±0.7	20.1±1.7	31.4±1.9	38.3±0.9
C57BL/6J-Bg^d(H-2^b)	1.1±0.2	1.5±0.1	1.6±0.1	2.3±0.1	3.3±0.1	4.8±0.2
A/J(H-2^a)	1.3±0.1	1.3±0.2	0.7±0.2	4.6±0.5	6.1±0.8	7.0±0.9

[a]Groups of 6 to 8 mice were assayed 24 hr after treatment.
[b]All values are significantly greater ($P < 0.01$, Student's t-test) than saline-injected controls.

TABLE 2

The Sensitivity of Tumor Target Cells to $MnCl_2$-Stimulated NK Cell Activity in C57BL/6J Mice

	$\bar{x}$ % ^{51}Cr Release[a]	
Target Cell	Saline	$MnCl_2$ (40 μg/g)
YAC-1	17.0	30.6
EL4	4.1	7.8
P815	3.5	7.7
RBL-5	0.7	5.0

[a]Mean of 4 cultures at E:T ratio of 100:1, 24 hr after injecting C57BL/6J mice intramuscularly with $MnCl_2$.

TABLE 3

The Influence of Exposure Route on $MnCl_2$-Stimulated NK Cell Activity in C57BL/6J Mice

		$\bar{x}$ % ^{51}Cr Release ± SE		
Treatment (dose)[a]	Route[b]	25:1[c]	50:1	100:1
Saline	i.m.	8.1±0.8	12.5±1.3	15.5±1.4
$MnCl_2$ (40 μg/g)	i.m.	19.3±1.4[d]	30.5±2.0[d]	37.6±2.7[d]
$MnCl_2$ (40 μg/g)	i.p.	19.3±2.6[d]	27.5±2.3[d]	35.0±2.9[d]
$MnCl_2$ (40 μg/g)	s.c.	19.5±0.8[d]	29.9±1 4[d]	34.2±2.1[d]
$MnCl_2$ (10 μg/g)	i.v.	14.3±1.3[d]	22.9±2.5[d]	30.1±2.7[d]
$MnCl_2$ (160 μg/g)	p.o.	6.6±0.7	11.7±1.1	16.0±1.6

[a]Groups of 6 C57BL/6J mice were treated 24 hr before assay.
[b]Subcutaneous (s.c.), oral gavage (p.o.), intraperitoneal (i.p.).
[c]Effector:target cell ratio, YAC-1 target.
[d]$P < 0.01$, Dunnett's t-test compared with saline intramuscular injection.

TABLE 4

The Effect of Phagocytic and Nylon Wool-Adherent Cell Removal on NK Cell Activity of $MnCl_2$-Injected C57BL/6J Mice

In Vivo Treatment[a]	In Vitro Treatment	% Cells Recovered	$\bar{x}$ % ^{51}Cr Release ± SE[b] E:T Ratio 25:1	50:1	100:1
Saline	None	100	7.6±0.8	11.5±1.0	15.5±0.9
	Carbonyl iron	59	11.4±0.6	14.0±0.9	17.1±0.5
	Carbonyl iron and nylon column	10	16.3±2.0	17.3±1.9	21.0±1.5
$MnCl_2$	None	100	14.4±1.4[c]	23.1±2.1[c]	34.1±1.0[c]
	Carbonyl iron	43	18.0±2.1[c]	25.6±1.8[c]	36.5±1.9[c]
	Carbonyl iron and nylon column	8	30.7±1.1[c]	38.9±1.7[c]	44.7±2.2[c]

[a]Groups of 5 mice were injected intramuscularly with saline or 40 μg/g $MnCl_2$ 24 hr before assay.
[b]Values represent the means of 3 replicate experiments with YAC-1 targets.
[c]$P < 0.01$, Student's t-test saline vs. $MnCl_2$.

from saline- and $MnCl_2$- treated mice gave only 10 and 8% recoveries, respectively, an approximately twofold increase in cytotoxicity was achieved by the fractionation in both cases. These results indicate that the major cytotoxic activity of spleen cells can be recovered in the nonadherent and nonphagocytic cell fractions after CI and CI-NW treatments and that $MnCl_2$-enhanced cytotoxicity is primarily associated with these cell fractions.

Further characterization of $MnCl_2$-enhanced cell populations was achieved by injecting mice with rabbit anti-asialo GM_1 serum (Table 5). Anti-asialo GM_1 serum (0.1 mg protein/mouse) significantly suppressed the cytotoxic activity in saline- and $MnCl_2$-injected mice compared with saline- and $MnCl_2$-injected mice treated with normal rabbit serum. $MnCl_2$ + normal serum mice had significantly enhanced activity compared with saline + normal serum mice. Therefore, $MnCl_2$ stimulated cytotoxic activity of cells that were asialo GM_1 positive, nonphagocytic, and nylon wool nonadherent, which strongly suggests that the effector cells are NK cells.

The role of IFN in $MnCl_2$ enhancement of NK cell activity was studied by measuring IFN levels in serum of $MnCl_2$-injected mice and by treating $MnCl_2$-injected mice with anti-IFN antibody. Figure 1 shows the kinetics of serum IFN levels and NK activity in C57BL/6J mice as a result of a single $MnCl_2$ injection. Within 4 hr after an intramuscular injection of 40 mg/g $MnCl_2$, IFN levels (43 IU/ml) were detectable in serum. Interferon levels peaked 16 hr after $MnCl_2$ injection (60 IU/ml) and then returned to baseline (10 IU/ml,

TABLE 5

The Inhibition of $MnCl_2$-Stimulated NK Cell Activity by Rabbit Anti-Asialo GM_1

Treatment[a]	$\bar{x}$ % ^{51}Cr Release ± SE E:T Ratio		
	25:1	50:1	100:1
Saline + Normal Serum	8.9±0.8	14.5±1.2	17.0±1.2
Saline + Anti-Asialo GM_1	1.1±0.6[b]	2.1±0.3[b]	1.7±0.4[b]
$MnCl_2$ + Normal Serum	16.5±1.3	26.8±1.4	32.7±1.6
$MnCl_2$ + Anti-Asialo GM_1	1.7±1.1[b]	1.1±0.3[b]	1.3±0.3[b]

[a]Groups of 8 C57BL/6J mice were injected intravenously with normal rabbit serum (1:10 dilution) or rabbit anti-asialo GM_1 (0.1 mg protein/mouse) followed by an intramuscular injection of saline or 40 μg/g $MnCl_2$ 24 hr before assay with YAC-1 targets.
[b]$P < 0.01$, Student's t-test, compared with normal serum controls.

the limit of sensitivity) by 48 hr. NK activity was significantly enhanced within 16 hr, peaked at 24 hr, and remained elevated for 4 days following $MnCl_2$ treatment. Enhanced NK cell activity persisted 3 and 8 days after $MnCl_2$ treatment in CBA/J and A/J mice, respectively (data not shown).

Comparable NK activity was observed for $MnCl_2$- and poly I·C-injected mice 16 hr after treatment (Table 6). NK activity was significantly enhanced at 25 to 100 μg poly I·C/mouse and at 20 to 80 μg/g $MnCl_2$.

Serum IFN levels 16 hr after $MnCl_2$ or poly I·C treatment were significantly different. For example, while NK activity was equal following treatment with 50 μg/mouse poly I·C or 40 μg/g $MnCl_2$, the IFN titers were 136 IU/ml and 16 IU/ml, respectively. Mice

TABLE 6

Comparison of IFN Titer and NK Cell Activity in Poly I·C- and $MnCl_2$-Treated C57BL/6J Mice

Treatment[a] (dose)	IFN Titer[b] (IU/ml)	$\bar{x}$ % ^{51}Cr Release ± SE[c] E:T Ratio 25:1	50:1	100:1
RPMI	<10	7.3±0.6	10.9±0.8	14.2±0.7
Poly I·C (25 μg)	77	16.9±0.7[c]	26.2±1.0[c]	33.7±0.9[c]
Poly I·C (50 μg)	136	17.4±1.1[c]	26.7±1.4[c]	35.5±1.5[c]
Poly I·C (100 μg)	147	14.5±0.5[c]	22.4±0.9[c]	31.3±1.3[c]
Saline	<10	7.4±0.8	11.2±1.4	13.9±1.1
$MnCl_2$ (20 μg/g)	<10	15.2±2.5[c]	23.7±3.7[c]	32.0±3.3[c]
$MnCl_2$ (40 μg/g)	16	17.3±1.6[c]	27.3±2.6[c]	36.1±2.9[c]
$MnCl_2$ (80 μg/g)	80	17.4±0.9[c]	27.6±1.7[c]	38.2±1.5[c]

[a]Groups of 6 C57BL/6J mice were injected intravenously with RPMI medium or Poly I·C in RPMI on a per mouse basis or intramuscularly with saline or $MnCl_2$ on a body weight basis 16 hr before assay with YAC-1 targets.
[b]Mean IFN titer of 6 mice per group.
[c]$P < 0.01$, Dunnett's t-test with RPMI vs. Poly I·C and saline vs. $MnCl_2$.

injected with 20 μg/g $MnCl_2$ showed significant enhancement of NK activity in the absence of detectable IFN levels. Either very low levels of IFN are required for NK enhancement or $MnCl_2$ enhances NK activity by mechanisms other than IFN induction alone.

Studies using rabbit anti-mouse IFN α,β or γ provided evidence that IFN α,β mediates $MnCl_2$ enhancement of NK cell activity. Table 7 shows the results from two of four studies in which mice were injected with either anti-IFN α,β or anti-IFN γ 1 hr before and 7 hr after a single intramuscular $MnCl_2$ injection. Saline-treated mice injected with either normal rabbit serum or

TABLE 7

The Elimination of $MnCl_2$-Stimulated NK Cell Activity by Rabbit Anti-Mouse IFN

Treatment[a]	$\bar{x}$ % ^{51}Cr Release ± SE E:T Ratio 25:1	50:1	100:1
Saline	6.5±1.1	9.4±0.9	13.1±0.9
Saline + Normal Serum	7.7±0.7	11.7±1.1	14.9±0.9
Saline + Anti-IFN α,β	6.9±1.3	11.9±0.8	16.8±1.1
$MnCl_2$	17.3±1.4	27.6±1.4	38.7±1.8
$MnCl_2$ + Normal Serum	14.7±1.6	23.8±3.3	30.3±3.8
$MnCl_2$ + Anti-IFN α,b	7.5±1.3[b]	12.0±1.3[b]	15.1±1.6[b]
Saline	7.2±0.6	10.4±0.8	13.3±0.9
Saline + Normal Serum	5.7±0.4	8.5±0.7	9.8±0.6
Saline + Anti-IFN γ	6.9±0.6	10.4±0.6	12.8±0.7
$MnCl_2$	19.7±0.7	30.6±1.1	38.9±1.4
$MnCl_2$ + Normal Serum	17.4±0.8	26.1±1.5	33.2±1.4
$MnCl_2$ + Anti-IFN γ	16.8±1.6	25.9±2.6	31.8±2.5

[a]Groups of 8 C57BL/6J mice were injected intravenously with rabbit anti-mouse IFN α,β serum (1,000 neutralizing units), anti-mouse IFN γ (7,000 neutralizing units), or normal rabbit serum (1:10 dilution) 1 hr before and 7 hr after a single intramuscular injection of saline or 40 μg/g $MnCl_2$. Spleen cells were assayed with YAC-1 targets 24 hr after $MnCl_2$ treatment.

[b]$P < 0.01$, Dunnett's t-test, comparing $MnCl_2$ vs. $MnCl_2$ + normal serum and $MnCl_2$ + anti-IFN α,β serum.

rabbit anti-mouse IFN α,β, or γ had NK activity comparable to that of saline-only injected mice. The NK activity of mice injected with $MnCl_2$ only displayed the expected two to three fold increase above saline-only injected mice. However, the NK activity of $MnCl_2$-treated mice injected with rabbit anti-mouse IFN α,β was reduced to that of saline-injected mice. Rabbit anti-mouse IFN γ

did not reduce $MnCl_2$-enhanced NK activity anymore than normal rabbit serum. The slight decrease in $MnCl_2$ + normal serum-injected mice may be due to the stress associated with the handling of these mice during the two intravenous injections, which probably accounts for the slight decrease in NK activity in $MnCl_2$ + anti-IFN γ compared with $MnCl_2$-only injected mice. These results indicate that IFN mediates $MnCl_2$ enhancement of NK activity *in vivo*.

$MnCl_2$ suppresses tumor development. The B16-F10 lung tumor model system was used as an *in vivo* correlate of $MnCl_2$-enhanced NK cell activity (Table 8). Mice were injected intramuscularly with $MnCl_2$ 24 hr before B16-F10 melanoma cell injections. Tilorone-treated mice served as positive controls. Lung tumor development was significantly suppressed in mice injected with 20 μg/g $MnCl_2$, and the number of tumor colonies was reduced in mice treated with 10 and 15 μg/g $MnCl_2$ 15 days following tumor cell injection. The $MnCl_2$-induced suppression of B16-F10 lung tumors was observed in three additional experiments (data not shown). Mice treated with tilorone, a known IFN inducer and NK enhancing agent, also had significantly fewer lung tumors than saline controls.

DISCUSSION

We have shown that a single parenteral injection of $MnCl_2$ enhances murine splenic CMC, which exhibits many of the characteristics of NK cells. The $MnCl_2$-enhanced activity is similar to that displayed by more complex agents that enhance NK activity through IFN production (9,10,11,19).

TABLE 8

Suppression of B16-F10 Lung Tumors in $MnCl_2$-Injected C57BL/6J Mice

Treatment (dose)[a]	$\bar{x}$ Tumors/Lung ± SE
Saline	47.5±10.1
$MnCl_2$ (10 μg/g)	24.8± 7.1
$MnCl_2$ (15 μg/g)	31.9± 7.0
$MnCl_2$ (20 μg/g)	9.0± 1.5[b]
Tilorone (2 mg)	6.5± 2.6[b]

[a]Groups of 10 C57BL/6J mice injected intramuscularly with saline or $MnCl_2$ or given tilorone orally 24 hr before an intravenous injection of 1×10^5 B16-F10 melanoma cells.
[b]$P < 0.001$, Mann-Whitney U test.

The $MnCl_2$-induced effector cells in the 4-hr ^{51}Cr release assay are nonphagocytic, nylon wool nonadherent, and possess surface markers for anti-asialo GM_1, all of which are characteristic of NK cells (15,16). Manganese chloride enhances NK cell activity in several mouse strains that have been shown to have varying NK reactivity (3). It also enhances the lytic capacity of splenic NK cells against several targets shown to have varying NK sensitivity (17,18). IFN α,β mediates $MnCl_2$ enhancement of NK cell activity. Although IFN levels returned to normal within 48 hr, the enhancement persisted for up to several days after a single injection. The complete elimination of NK cell enhancement in $MnCl_2$-injected mice treated with rabbit anti-mouse IFN α,β but not anti-IFN γ is further evidence for IFN's intermediary role in $MnCl_2$-enhanced NK activity. Manganese chloride enhancement of NK

cell activity can also protect against the growth of B16-F10 melanoma lung tumors, which is a characteristic of NK cells consistent with other studies (7,8).

Interferon and IFN inducers stimulate NK activity in mice (9-11). The effect of IFN and IFN inducers on NK cell activity appears to be an all-or-none response (19). As observed in the present study, increases in poly I·C- or $MnCl_2$-induced IFN did not result in further enhancement of the cytotoxic activity of the splenocytes (see Table VI). Similarly, the poly I·C doses used here and the poly I·C and tilorone hydrochloride doses used in earlier work (13) stimulated IFN production more than $MnCl_2$, but comparable significant enhancement of NK activity was observed for all three of these agents. While these observations are consistent with an all-or-none response mediated by increased IFN levels, factors other than IFN may play a role in $MnCl_2$-enhanced NK cell activity. For example, $MnCl_2$ may induce the production of interleukin 2, which has been shown to affect murine NK cells (20). At present, however, we have no evidence for $MnCl_2$ induction of this or any other soluble immuno-modulating agent other than IFN α,β.

Macrophages also develop augmented cytotoxicity after exposure to IFN or IFN inducers (9,21,22). However, macrophages are not responsible for $MnCl_2$-enhanced CMC in the 4-hr ^{51}Cr release assay because: 1) the effector cells were nonphagocytic and nylon wool nonadherent and 2) the detection of macrophage cytotoxicity requires longer incubation times and cell lines (e.g. P815, L5178Y)

that are susceptible to macrophage-mediated cytolysis (17,18). We did, however, observe that $MnCl_2$ injection stimulated resident peritoneal macrophages. Specifically, macrophages from $MnCl_2$-treated mice had enhanced phagocytic capacity as well as enhanced cytotoxic and cytostatic capacity against tumor cells *in vitro* (manuscript in preparation). This enhanced macrophage function can be explained by the known effects of IFN on phagocytosis (23) and the tumoricidal capacity (22) of macrophages.

We do not know the mechanism of $MnCl_2$-stimulated IFN production. We believe that endotoxin does not play a direct or indirect role in the observed NK cell enhancement. The stock $MnCl_2$ solution used in these studies was free of endotoxin as assayed by the *Limulus* amebocyte lysate assay, (Sigma, St. Louis, MO). Enhanced NK activity was also observed in mice injected with $MnCl_2$ obtained from another source (Sigma, data not shown) and in C3H/HeJ mice (Table 1), which are refractory to NK enhancement by endotoxin (24). Furthermore, release of endotoxin from the gastrointestinal tract in response to $MnCl_2$ injection does not appear to be a mechanism because enhanced IFN and consequently enhanced NK cell activity were observed in 18 hr spleen cell cultures treated with $MnCl_2$ *in vitro* (manuscript in preparation). $MnCl_2$-induction of a latent viral infection is also an unlikely cause of increased IFN levels because the IFN titer rises rapidly following $MnCl_2$ injection (see Figure 1). Also, $MnCl_2$-injected mice did not have antibody titers to pneumonia virus of mice, reovirus Type 3, encephalomyelitis, K-virus, polyoma, minute virus

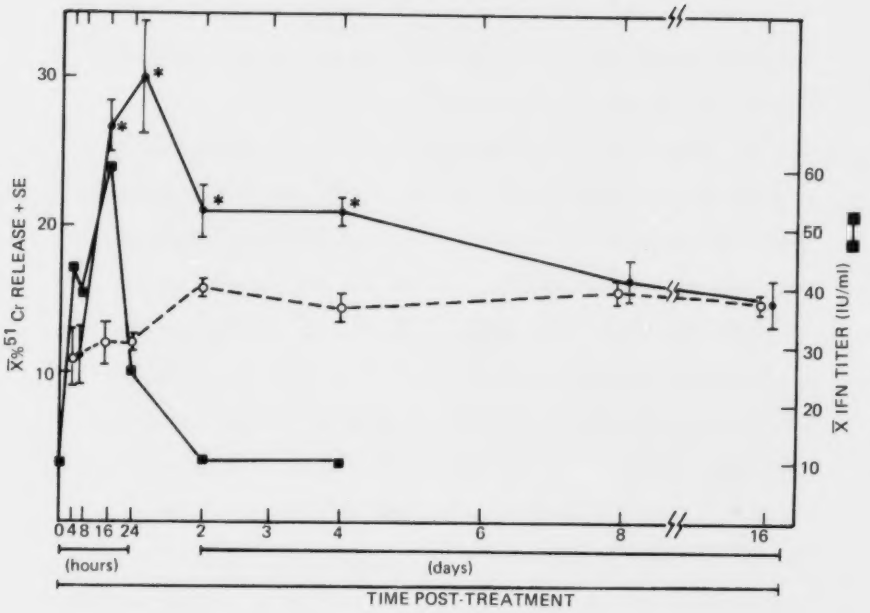

FIG. 1. The kinetics of $MnCl_2$-stimulated NK cell activity and IFN production. Groups of 6 C57BL/6J mice were given a single intramuscular injection of saline (0) or 40 μg/g $MnCl_2$ (●). Data are the mean percent ^{51}Cr release with YAC-1 targets at an effector:target cell ratio of 50:1. The asterisk indicates a $P < 0.01$, Student's test between saline- and $MnCl_2$-treated mice. Interferon levels (■) are the mean IU/ml for 6 mice.

of mice, sendai, mouse adenovirus, lymphocytic choriomeningitis, mouse hepatitis, and ectromelia (Biomedical Reference Lab., Inc. Burlington, NC). As mentioned above, in vitro culture of splenocytes with $MnCl_2$ induces IFN production and NK activity which further suggests that a host response to an activated viral infection is not a likely explanation for increased IFN and NK activity.

Manganese has diverse biochemical and physiological effects and is an essential trace element for man and animals (25-27). However, it is also toxic in sufficiently high doses. For example, chronic inhalation of manganese dusts can lead to manganism, which is primarly a disease of the central nervous system with symptoms similar to Parkinson's disease (28). Manganese is a nonspecific activator of numerous enzymes that require the presence of a divalent ion, while other enzymes specifically require the presence of manganese ions (29). Manganese also inhibits carcinogenesis (30,31) and cell transformation (32) by metallic and nonmetallic compounds, increases the potency of an anti-Meth A lymphosarcoma vaccine (33), and protects mice against endotoxin-induced mortality (34). We are unaware of any metal salt other than $MnCl_2$ that enhances NK cell activity in a manner similar to that of more complex molecules, i.e. through induction of IFN. Further work is required to determine the mechanism of manganese ion-induced IFN production.

DISCLAIMER

This report has been reviewed by the Environmental Protection Agency's Office of Research and Development, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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